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TITLE: Clinical Phase IIB Trial of Oxycyte Perflurocarbon in Severe Human Traumatic Brain Injury

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14. ABSTRACT

Cerebral ischemia is a common secondary consequence of traumatic brain injury (TBI), including penetrating TBI. Experimental models of closed brain injury (fluid percussion, subdural hematoma, etc.) have demonstrated a neuroprotective effect after perfluorocarbon (PFC) administration, however, there is no data relating to a pharmacological effect of PFC in penetrating ballistic-like brain injury (PBBI;Tortella model) that is characterized by acute, sustained significant hypoxia. In this report we present data on the severity of hypoxia, glucose utilization, histopathology and oxygen metabolism(Vo2) following PBBI, and changes in these parameters following PFC administration in the PBBI model, as well as studies on effect of PFC on blood clotting of normal human blood. The data shows little effect of any of the 3 PFC's on the hypoxia observed in this type of brain injury. In the same PBBI injury model for the first time we observed no effect of PFC's upon VO2 (oxygen consumption) but the studies are incomplete. Surprisingly, the 2 PFc's so far tested significantly improved Glucose utilization (anaerobic glycolysis) but the studies are incomplete. The. We are currently further investigating the effects of PFC administration on this PBBI induced global glucose utilization. The Cell counting study aimed at uncovering any neuroprotective effects of PFC administration on PBBI induced neurodegeneration did not reveal significant global improvement, but the counts are incomplete. Interestingly the three PFCs tested had very different effects on the thromboelastography profile of normal human blood. Only Perftec demonstrated an apparent anticoagulant effect, using Thromboelastography. This data will be used to assess the safety/efficacy profile of each PFC, in the PTBI and closed TBI models. About 60% of experiments are complete, and data analysis is ongoing.

15. SUBJECT TERMS

Perfluorocarbons

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Introduction

Perfluorocarbons are one of the methods by which oxygen delivery to tissue can be achieved after injury. Neurological injury [brain and cord] is always accompanied by tissue ischemia/hypoxia and much of the damage seems to be mediated by this secondary mechanism. The rationale for perfluorocarbons in traumatic brain injury has been well established in animal studies and early phase 2 clinical trials. Currently three perfluorocarbons are available in the United States for testing, but none of these have been FDA approved and only for one of them – Oxycyte has the process of application for FDA approval even been commenced. For the third generation perfluorocarbon (Oxycyte) a possible side effect that has emerged in humans is transient mild thrombocytopenia. It is uncertain at this time whether this side effect will prove to be a limiting factor, which may jeopardize the use of these compounds as a class, or just affect Oxycyte in particular following traumatic brain and spinal cord injury. Any agent that might exacerbate thrombocytopenia, turn in intracranial hemorrhage into traumatic contusions could for obvious reasons be dangerous. The purpose of this grant therefore is to cross compare the safety and efficacy of three perfluorocarbons namely oxycyte, perftoran and oxygent. We will assess these 3 PFC agents in a new PENETRATING brain injury animal model, devised at WRAIR (the Tortella PTBI model) and in closed severe rat TBI with a secondary insult, for the first time, with such agents.

Body

The 4 specific aims are stated below:

Aim 1: PFC will be effective in mitigating penetrating TBI, as tested in the WRAIR/Tortella model of penetrating ballistic-like brain injury (PBBI), with acute brain histology, at 24 and 72 hours after injury, in the rat.
Aim 2: A—two doses of PFC, given 24 hours apart will be safe and effective in mitigating secondary ischemic damage, superimposed upon severe closed TBI in the rat.

2B--TEG will be performed in collaboration with the Wallace Coulter Platelet Function laboratory at the University of Miami
Aim 3. PFC's will improve both oxygen consumption ($CMRO_2$) and glucose use, in the rat brain, after TBI.
Aim 4. PFC will improve cell survival, in an in vitro model of mild TBI , when applied in the supernatant culture medium.

Our letter of award was made in August 2011 and in this **Fifth quarterly technical Progress report** we outline progress that has been made in the **3month period** from **July 1 2012—September 2012.**

(3) Section II - A brief description of overall progress to date plus a separate description for each task or other logical segment of work on which effort was expended during the report period. Description shall include pertinent data and graphs in sufficient detail to explain any significant results

achieved. If this award includes the recruitment of human subjects for clinical research or a clinical trial, report progress on subject recruitment (i.e., number of subjects enrolled versus total number proposed).

IACUC and ACURO Approvals
All University of Miami Institutional Animal Care and Use Committee [IACUC] approvals have now been received , and these new animal models and protocols have now **also been approved by the ACURO office, and all necessary radiation safety, and CITI certifications, for all personnel are in place.**
Personnel.
A medical student, Mr Khadil Hossein, has been recruited as a full time researcher to replace 2 students who left at end of June. He has been trained in Histology processing, and cell counting techniques, and animal surgery.

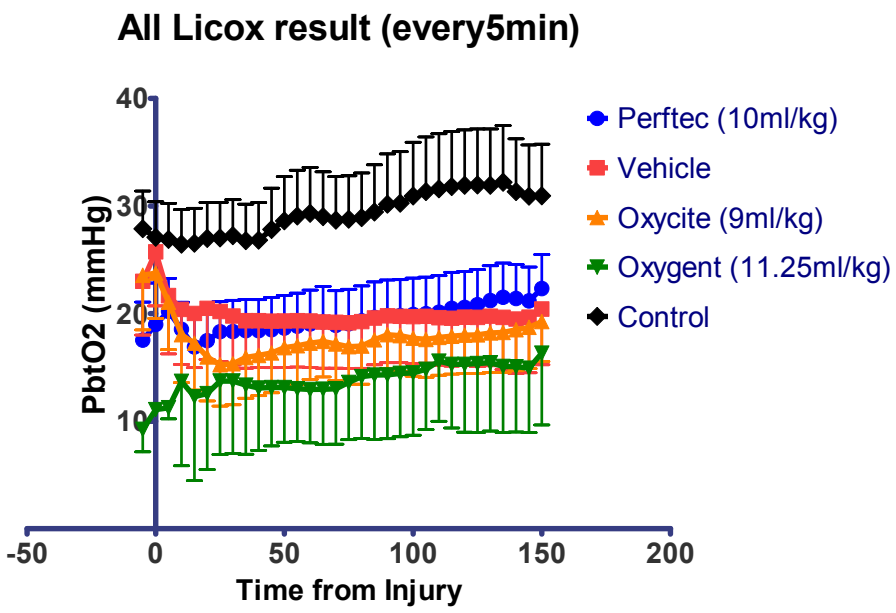
Table 1.-Overall Work summary table, for animal use.

Period	*Status of Animal use*	**Proposed In Grant**	**Completed To date**	**Remaining**	Comment
September 2011— September 2012					
Aim 1-PTBI	*100% complete*	50	50	0	Surgery is complete, histology in progress..60 % done
Aim2 TBI plus Hypoxia, and coagulation studies	*~45% complete*	60	24	26	TBI+hypoxia work with Oxycyte: TEG with PBBI, 60% complete, TEG in normal humans 60% complete.
Aim3 PFC and brain metabolism	*63% complete*	160	100	60	2DG studies and some VO2 studies with two PFCs and controls completed
Aim 4 PFC in mild/mod TBI-tissue culture	*75% complete*	20	15	5	Multiple cultures in progress
		290	189	101	
				35%	To be achieved

Key Research Accomplishments

Progress with Aim 1.
Oxygent PFC was obtained, in late april .We have now completed all rat surgery for aim 1 . Counts for FJB positive cells, is ongoing, and about 60% complete.
In addition, we have completed a series of animals to test the effect of the 3 PFC's on oxygen tension in the injured brain, using the LICOX system after PTBI. See abstract in appendix for results, and figure 1 below.

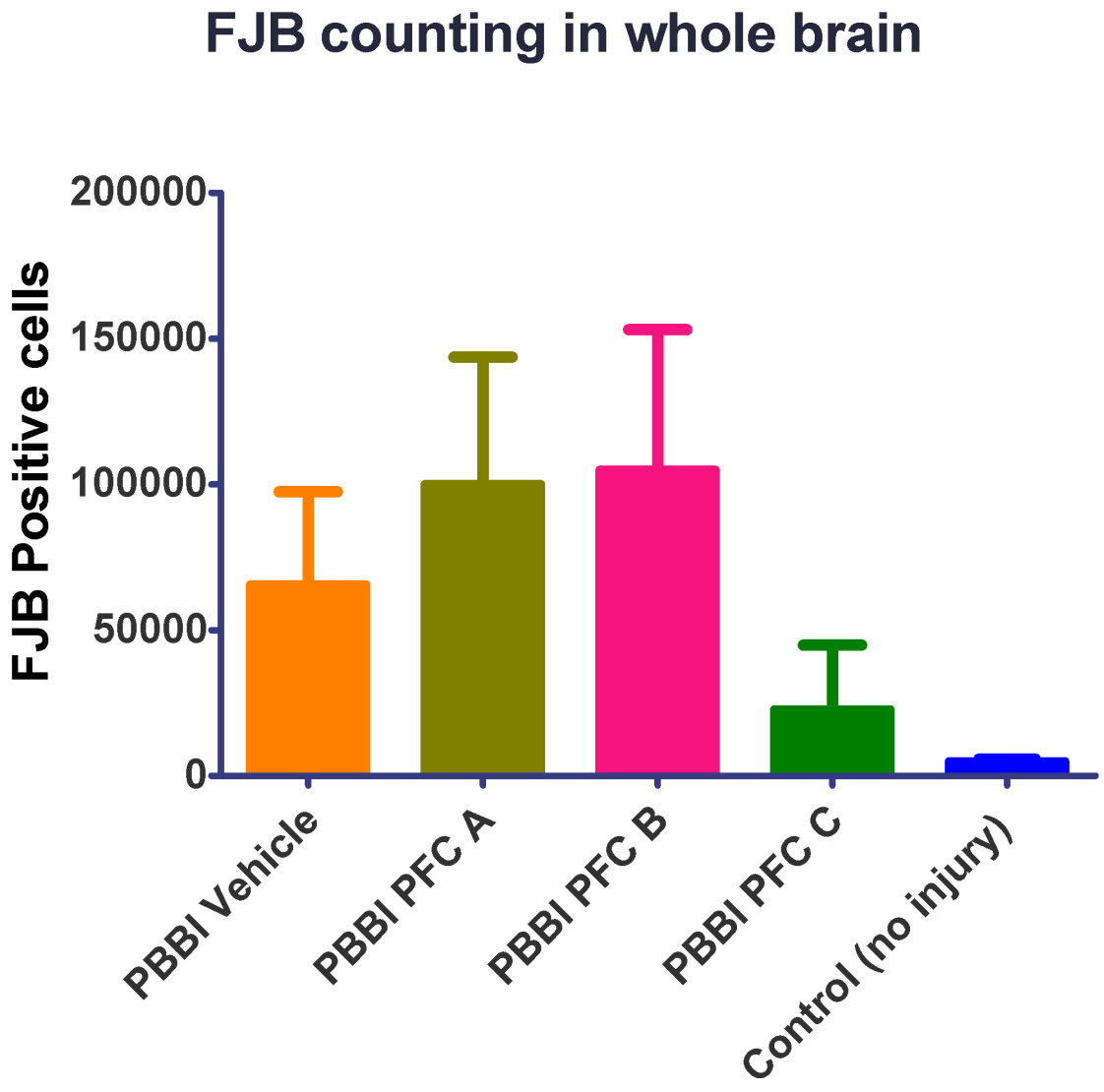
Figure 1.



Perftec was the most effective PFC for enhancing oxygen tension, in brain tissue as judged by Licox pBtiO_2 sensor. The magnitude of effect of Oxycyte and Oxygent was similar. **All 3 were similar to vehicle, at these high doses for this PTBI model. Surprisingly, no differences were significant from vehicle treated injured animals, with this severe PTBI model.**

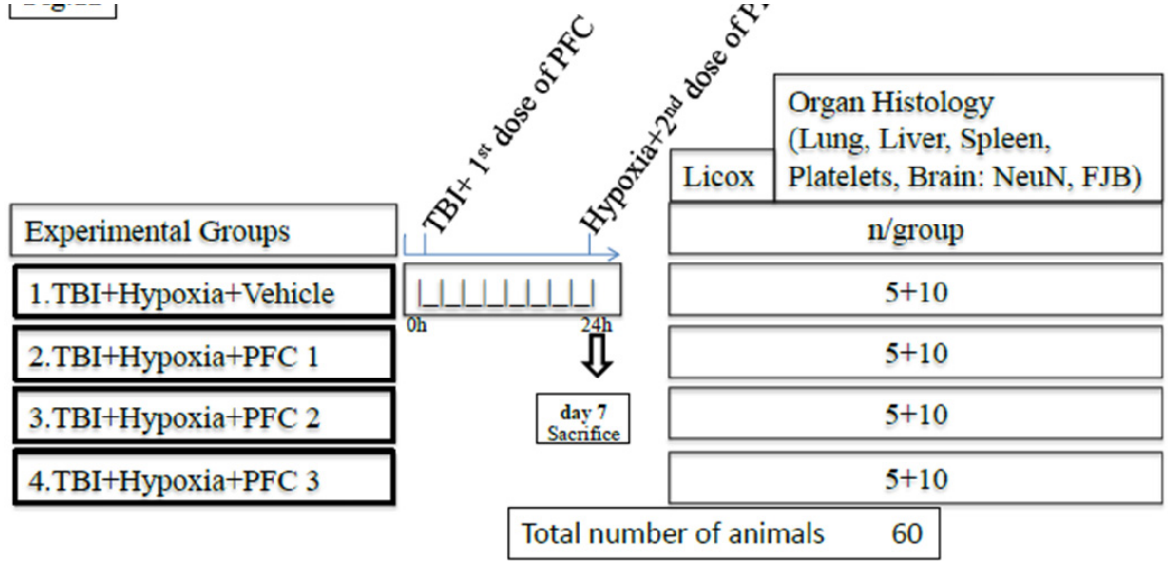
Ongoing cell counting for Fluoro –jade positive cells is being done, but counts require about 10 hours, per animal. **As the figure 2 below indicates, there is clear data separation, between the 3 PFC's tested, but since counting is incomplete, with N=4 per group, this data will change, and is therefore not designated by PFC name. This experiment is the most sensitive metric for a neuroprotective effect, for these PFC's, and the finalized, complete data from this aim 1 will be available by the next quarterly report.**

Figure 2


FJB counting in whole brain
FJB Positive cells
200000
150000
100000
50000
0
PBBI Vehicle
PBBI PFC A
PBBI PFC B
PBBI PFC C
Control (no injury)

Progress with Aim 2

Figure 3. Summary figure for aim 2.



The studies with Oxycyte, and secondary hypoxia, and controls are completed, (N=10 per group) and the studies with vehicle have enrolled 4. Perftec and Oxygent studies will be done over the next 4 months.

RESULTS

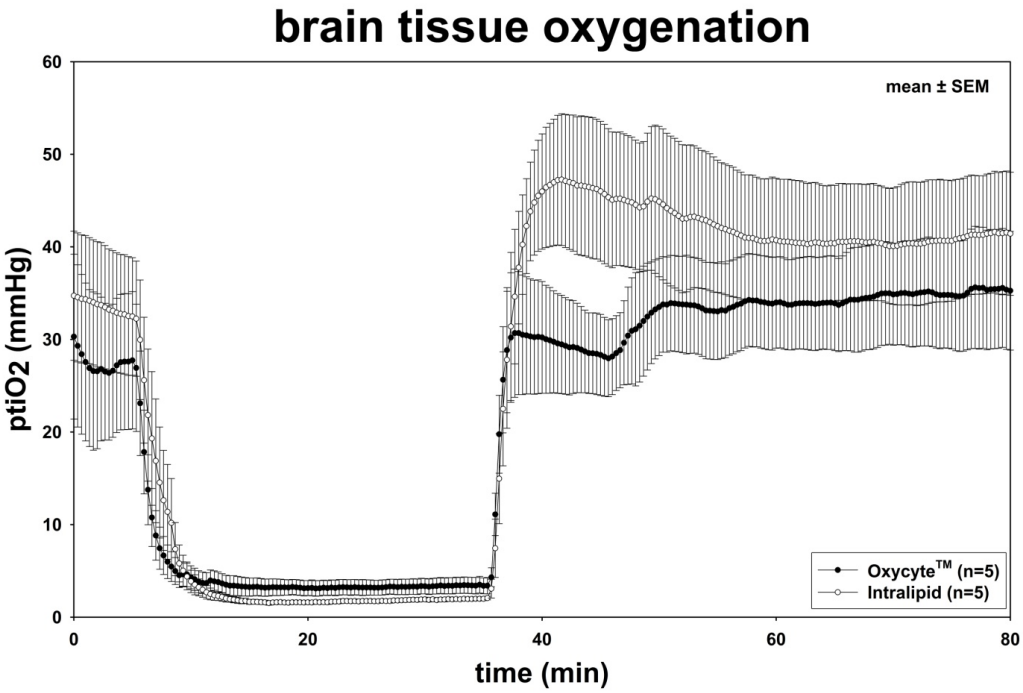


Figure 4 LICOX brain tissue oxygenation changes, during the secondary hypoxic insult, treated with the PFC Oxycyte, and with vehicle .

During the hypoxemic insult, eg at 20 mins, the PFC treated animals demonstrated higher brain PtiO2 values, compared to vehicle, and during the post –hypoxemic phase, eg 45 mins, the Oxycyte treated animals demonstrated less marked "rebound hyperoxia"…35mmhg vs 48 mmhg in the vehicle animals. None of these differences were significant.

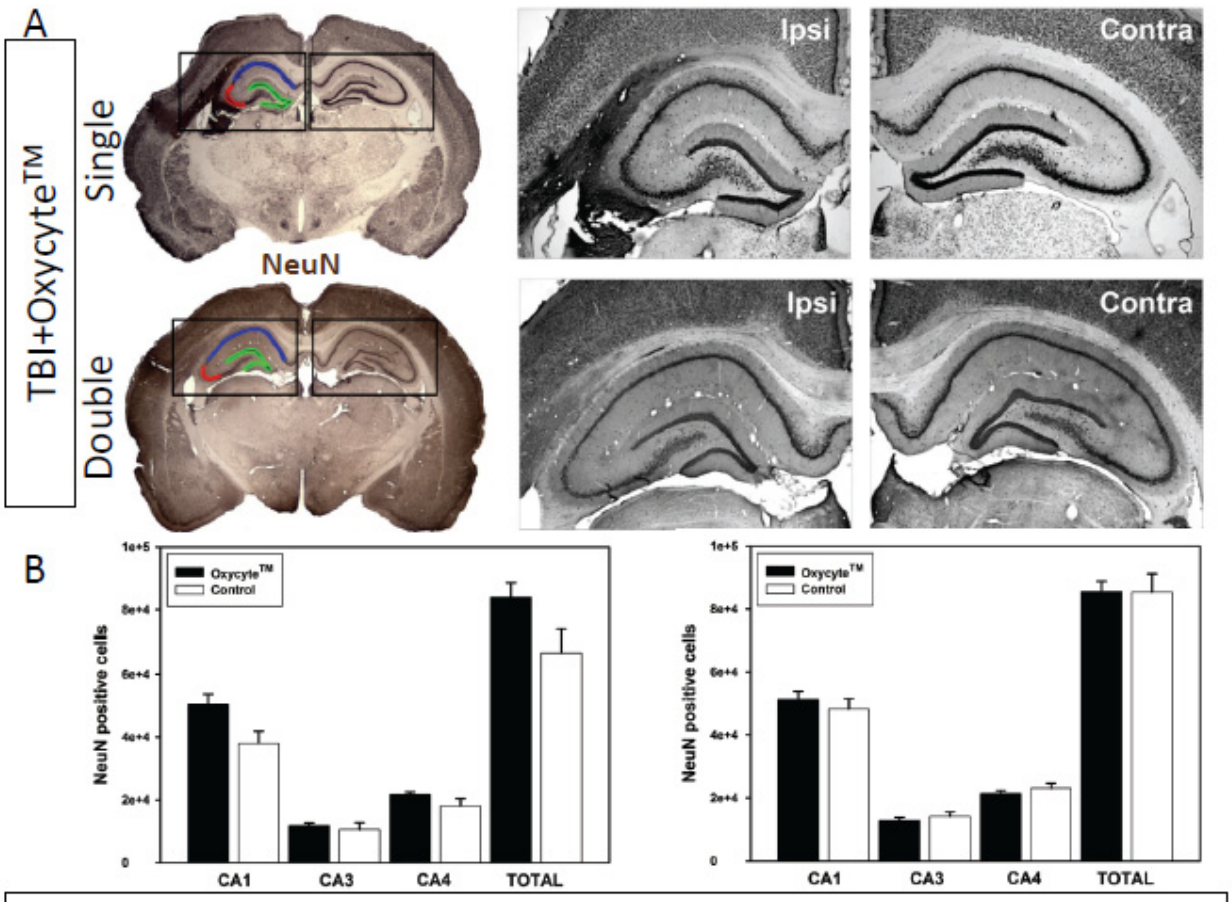


Figure 5. NeuN staining to test the effects of double dose Oxycyte administration on TBI–induced hippocampus cell loss. Images on left side in top panel (A) shows whole brain coronal sections with highlighted hippocampal areas. The boxed areas are shown at higher magnification on the right. Graphical representation of the healthy neuron counts is shown in (B). At 24h (B-left)a clear trend towards improved neuroprotection in CA1 especially could be observed. For the 7 day group (B- right) no difference was seen between one and 2 dose Oxycyte groups.

These studies suggest that 2 doses of Oxycyte confer no more benefit than a single dose, in this closed TBI –Hypoxia paradigm. We will await the result of the studies with the other two PFC's.

Liver and Spleen Histology.

Preliminary Liver and spleen histopathology data is available, for the first 3 animals given 2 doses of Oxycyte. –see fig 6 below. With Hematoxylin and Eosin staining, no evidence of cell damage, or inflammation was seen.

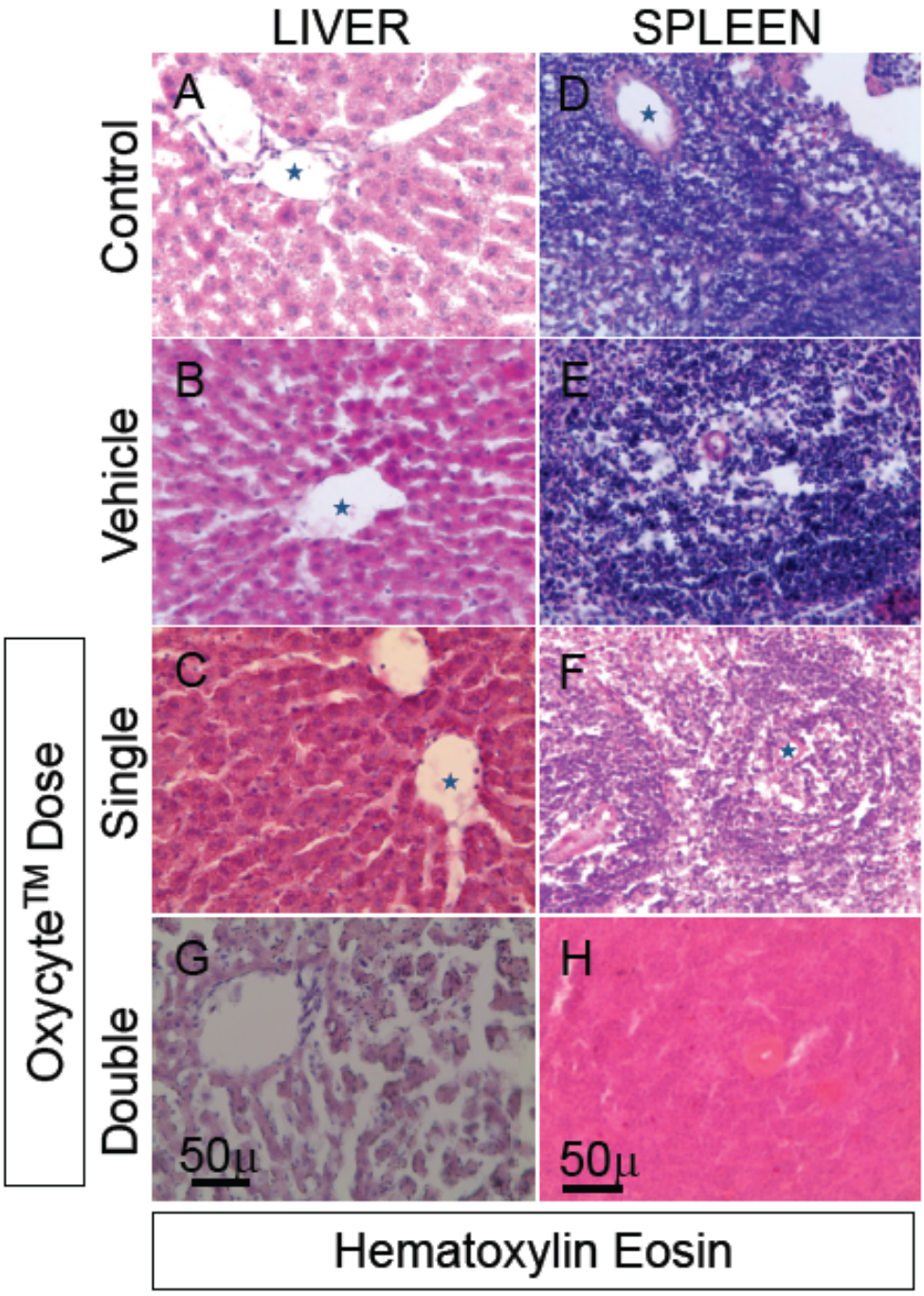


Fig. 6. No significant changes in the H&E stained structures were observed in the both doses of Oxycyte (C, G,F,H) compared to vehicle (B, E) treated livers (left panel) or spleen (right panel). The asterisk show well perfused empty blood vessels. Aggregates of thrombocytes or megakaryocyte or infiltrating phagocytes (indicators of inflammation and thrombocytopenia), were not observed in these tissues. All images are same magnification and scale bars are 50 microns.

TEG

The studies of coagulation in the presence of PFC's have been done in rats with Fluid percussion injury, and in Normal human blood, drawn from lab staff, and analysed within one hour of venesection.

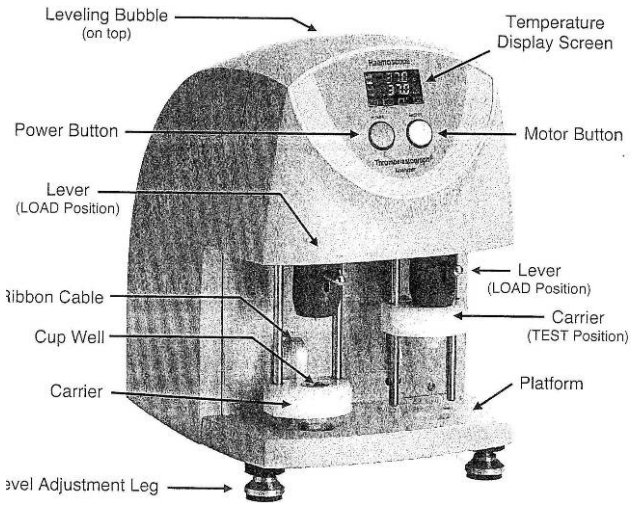


FIG 7 TEG coagulation analyzer

Results

As reported in the abstracts attached here (see Appendix), in both the normal human volunteers, and the TBI rats, **Perftec appears to have significant anticoagulant effect, possibly due to thrombolysis, while Oxygent and Oxycyte have no effect upon TEG parameters, in these experimental conditions, and doses , ie normal volunteer human blood.**

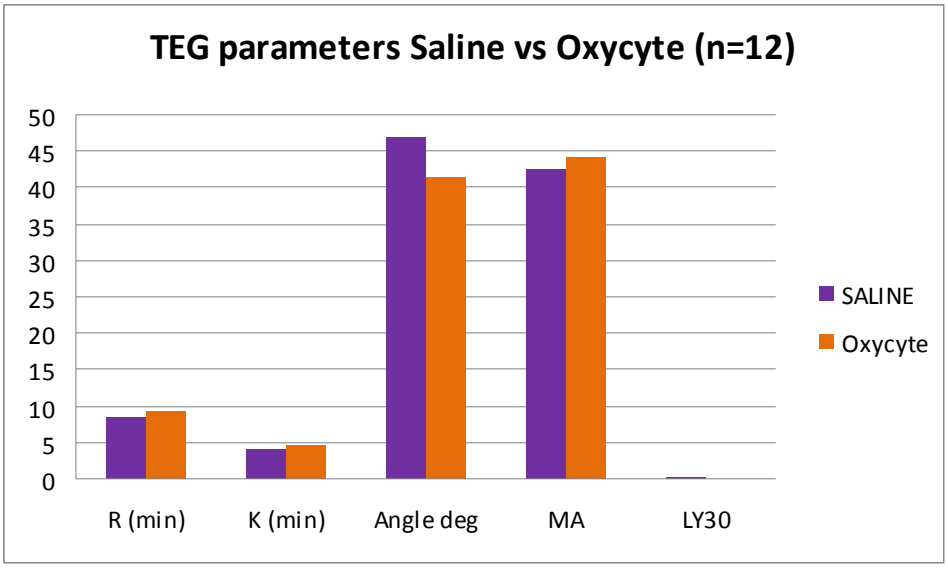


Fig 8 TEG data for Oxycyte….no significant differences from saline control. Dose =9ml/kg.

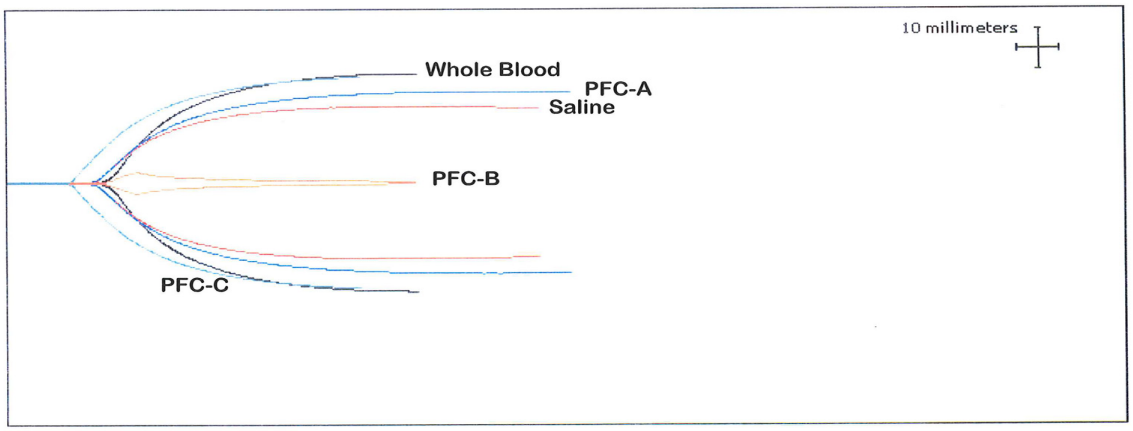


Figure 9 Averaged TEG tracings, for 3 PFC's…mean values A=oxycyte, B=Perftec, C=Oxygent.

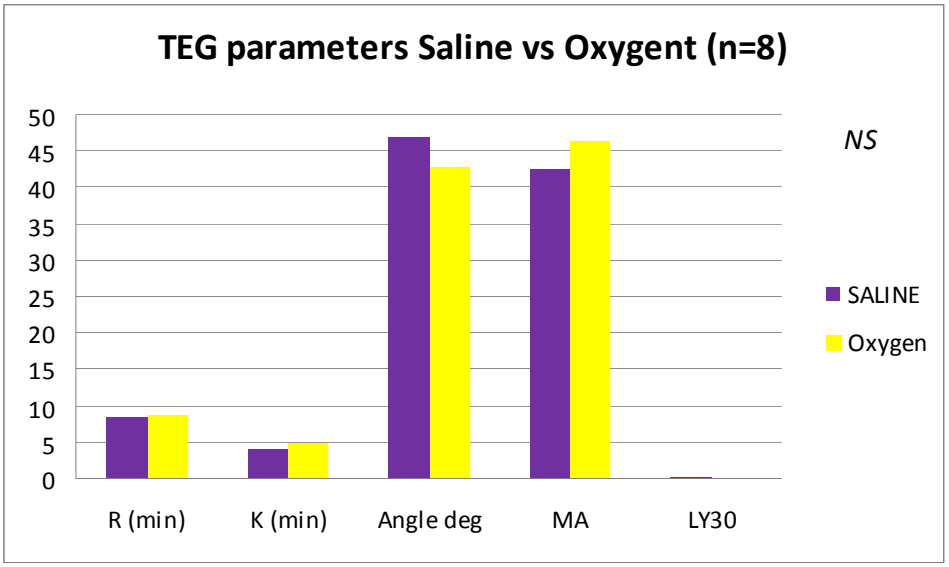


Figure 10. Effect of Oxygent, 10ml/kg, vs saline upon TEG parameters. No significant differences were seen.

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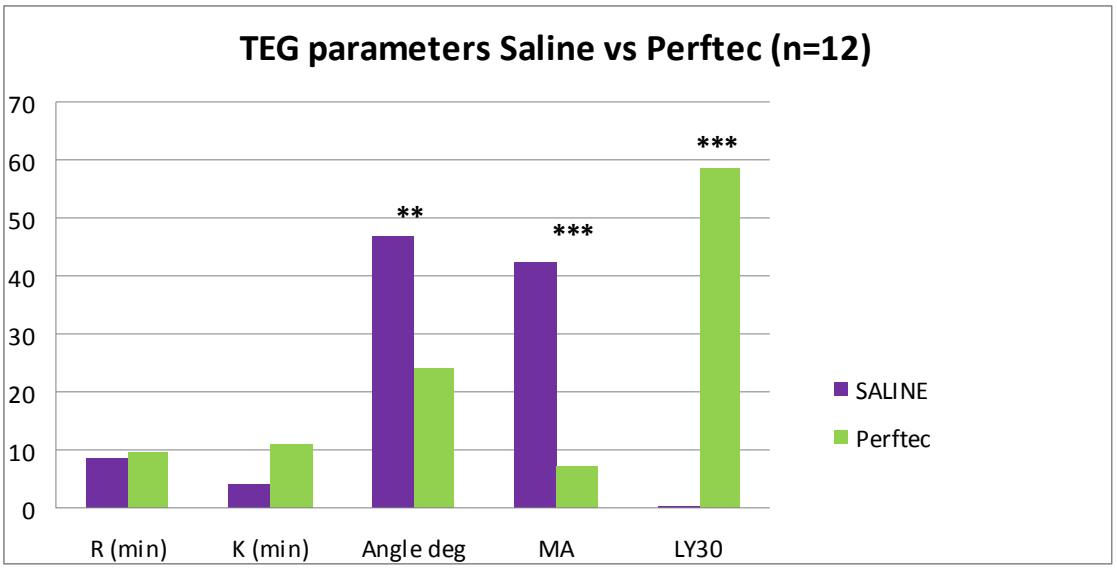

Fig 11. Effect of Perftec upon TEG coagulation parameters. 10 ml/kg. a significant anticoagulant effect was seen. ** P<0.05 *** P,0.001

This has major implications. If validated, in rats (ongoing) and TBI injured human blood, it suggests Perftec should not be used for TBI, based on the current data.

Rat blood studies with PTBI animals are almost completed.

Progress with Aim 3.

We have completed autoradiographic studies for 3 of the 4 groups, needed for part A of aim 3— see table 1. We have sectioned all these brains, and made the autoradiograms. –About 75% of the work. We will do the remaining "Oxygent" animals, in October. Preliminary analysis is shown below in fig 13.

The studies for aim 3 B –Microrespirometry, are nearly completed, for the PTBI model, see fig 14 below, for early data.. The Microrespirometry apparatus has been set up in Miami, and calibrated, and Dr Gajavelli is fully trained in its operation, currently.

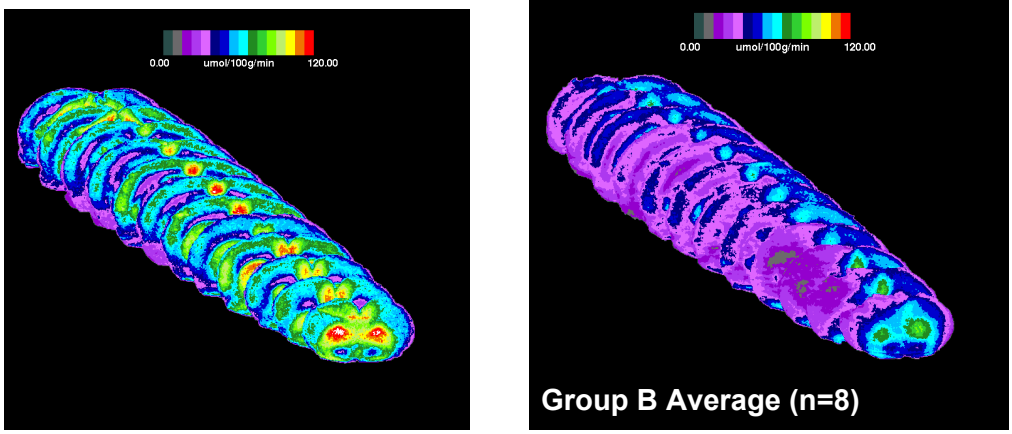

Figure 12—autoradiogram montage of Glucose use, in controls (left) and PTBI injured rat brain (right) Notice the marked whole hemisphere reduction, in the injured hemisphere, -values around 10% of normal.

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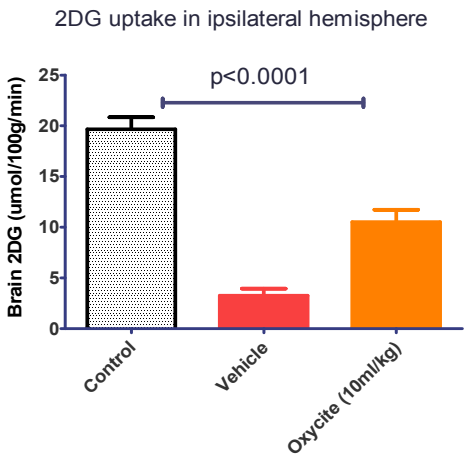


Figure 13. Glucose use, after PBBI, with oxycyte, note that glucose use is reduced about 10 fold, by the PBBI injury (vehicle) and this appears to be ameliorated significantly, by Oxycyte (approx 3 fold improvement) data for the other 2 PFC's is awaited.

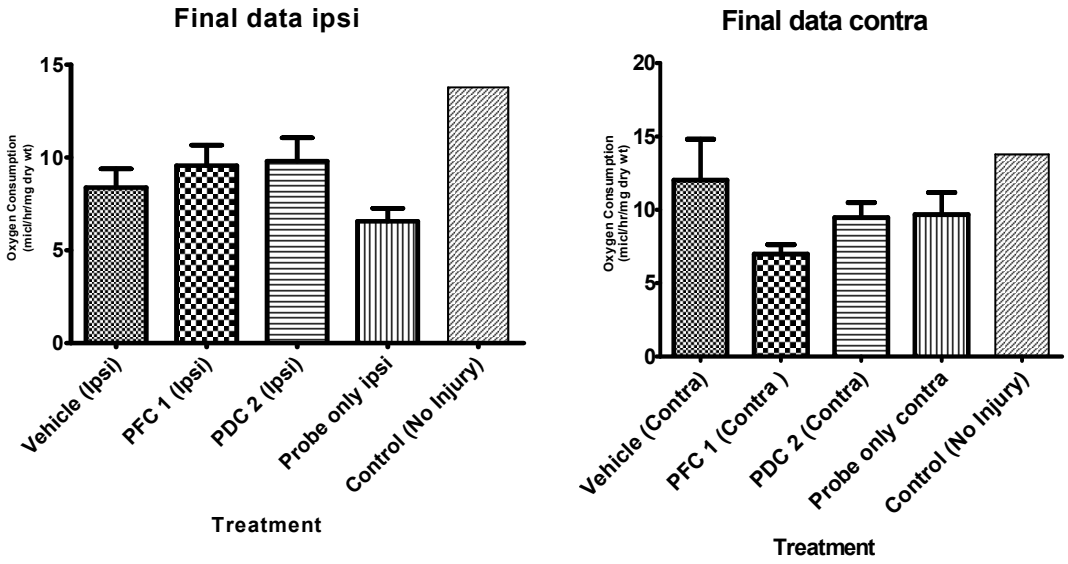
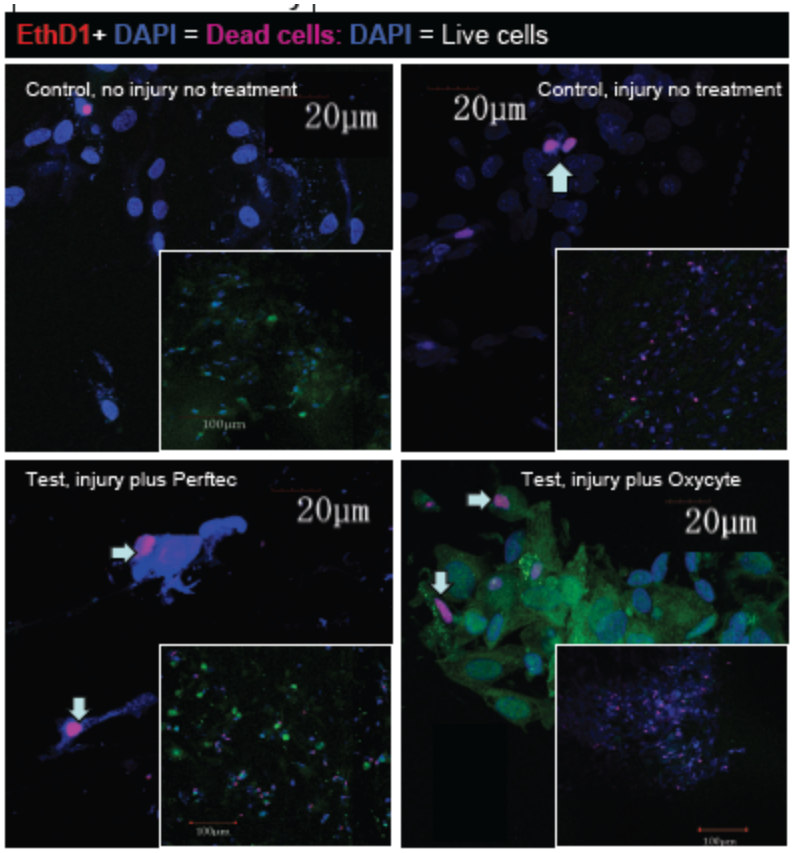


Figure14. Effect of 2 PFC's on VO_2 (brain tissue oxygen consumption,) after PBBI. No apparent robust improvement effect was seen, but these studies represent only 4 animals per group….

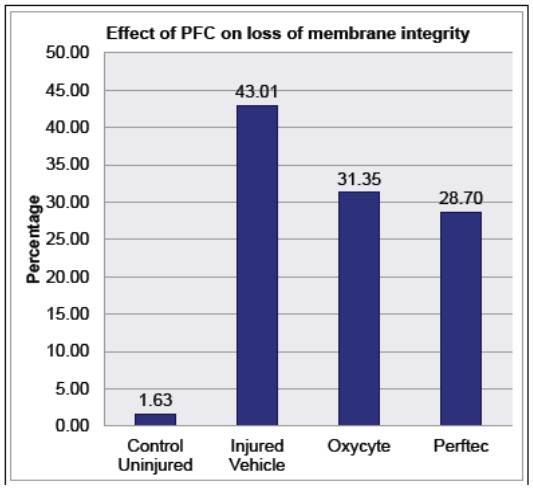
Progress with aim 4.

Figure 15. We have established the Pneumatic stretch well –plate model, to mimic mild TBI. we have established densely adherent monolayer mixed astrocyte/neuronal cultures, on the silastic membrane wells, and injured them, to produce cell death, as detected quantitatively, with Ethidium dibromide staining(shown red in panel 2 , figure 3 below. We have grown these cells in presence of a supernatant with 10 % added PFC emulsions, for the two PFC's we have at present. We have a single replicate experiment completed, with each PFC and saline controls, after stretch injury, and counts are ongoing, for that experiment. We now need to perform 5-6 replicates for each condition, and complete the counts. .



Images of four experimental groups after assay. Dead cells appear shades of purple (arrows) due to combined fluorescence of EthD-1 (red) and nuclear counter-stain DAPI (blue). The inset shows low magnification image of the cultures. Live cell stain (green) was washed off unevenly. The scale bar shows magnification.

Figure 16. The quantitation of the above groups showed that ~43% of cells lost membrane integrity following injury. The presence of PFCs Oxycyte and Perftec reduces this number to 31.5 and 28.7 respectively. This is an important early finding, which, if validated in more replicates, may suggest an intrinsic membrane stabilizing effect, of these large PFC molecules.



(4) Section III - Problem Areas
(a) A description of current problems that may impede performance along with proposed corrective action.

Pitfalls and problems.

Negative studies….*In Vitro* validation.

In response to the surprising absence of any apparent effect of any of the PFC's studied, upon VO_2 (fig 13 above) we decided to perform a simple *in vitro* experiment, to determine whether the supplied product which we have purchased, from the 3 vendors, was biologically active, at the physiological dose concentrations which we are using. For this, we placed a 10% solution of the PFC in saline, in a vial, and bubbled 100% oxygen into the vial, to mimic lung oxygenation of blood. We excluded red cells to focus on the theoretical strength of PFC—its ability to dissolve gases in the plasma phase, which in our view offers the major advantage for TBI treatment. We detected changes in dissolved oxygen, using the LICOX device, which continually detects PO2—oxygen tension, or partial pressure.

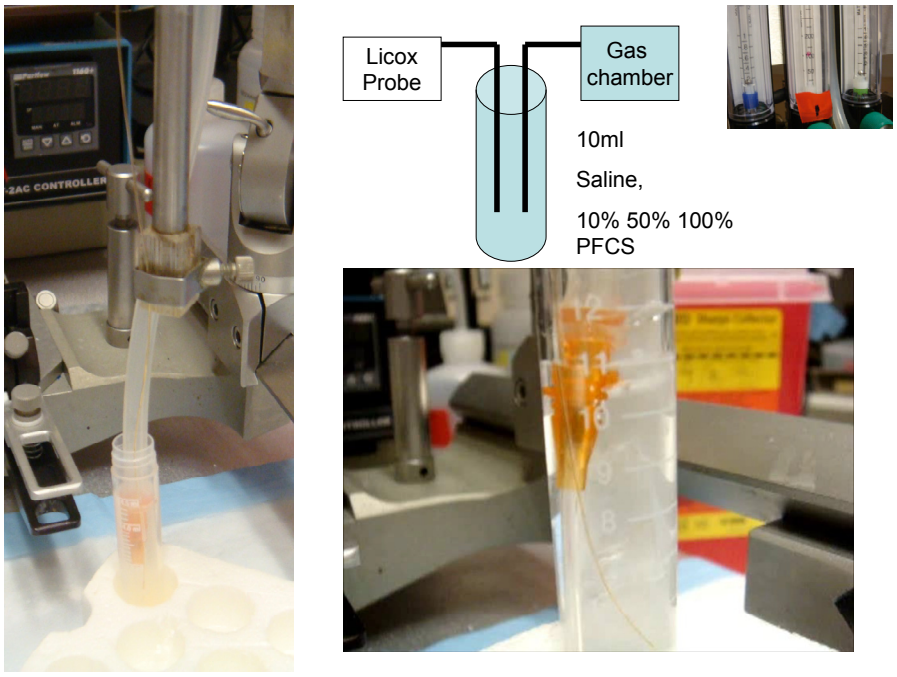


Figure 17. *In vitro* experiment setup.

Results.

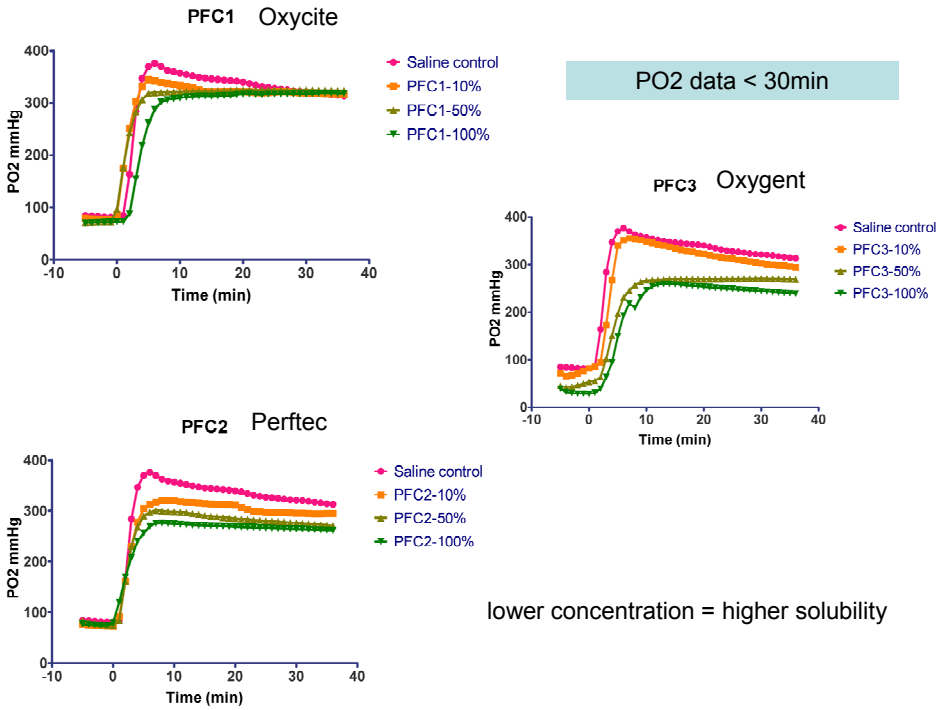


Fig 18. None of the PFC's studied showed any advantage over simply bubbling 100% oxygen alone, into saline, for the first 30 mins after oxygenation. The 10% concentration seemed better than 100% in each case.

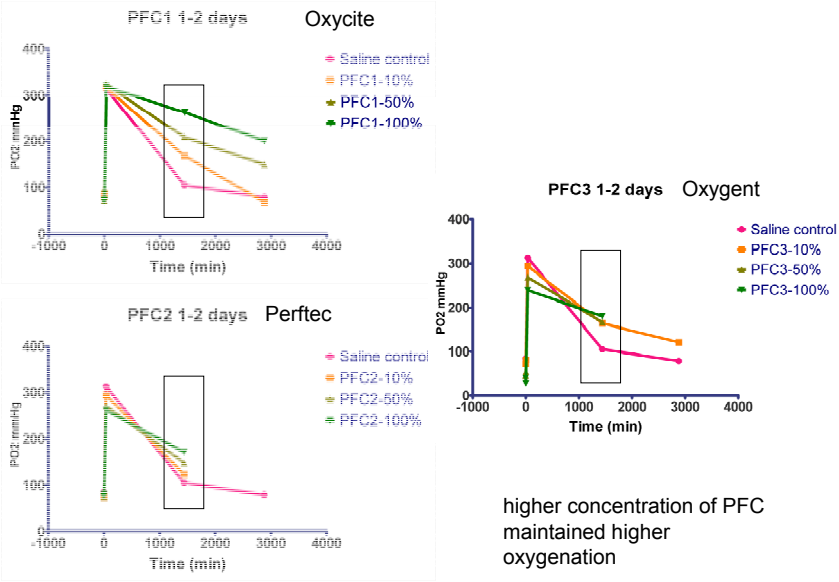


Figure 19 as time passes, and diffusion reduces PO2 in saline, to ambient (~100mmHg), the PFC's all show ability to retain more oxygen. The best effect was now seen at highest concentrations, (1005, and 50%) but these are not safe for clinical use. At 10%, Oxycyte showed the best effect, at about 24 hours (~170 mmHg).

These data show the supplied PFCs are active, but further *in vitro* studies, using blood, at 37degC, at 2-15 hours after administration, would be interesting, to get a real "feel" for the potency of the 3 agents, in comparison.

1) **Unavailability of the PTBI apparatus from WRAIR.** We are grateful to Dr Tortella and his group, for the loan of the PTBI apparatus. We have returned the apparatus, and are thus unfortunately not able to make up further experiments, if we find later that we need more replicate animals, etc.

2) **Availability of PFC's.** We have now been able to take delivery of Oxygent, and studies are ongoing. We required to purchase 2 more bottles of Oxycyte, at a total cost of $900 for ~200 ml.

3) **Cartesian Diver VO2 device.** —The "Cartesian Diver" device,is in regular use, and working well.

4) **Quality of Histological sections, for cell counts, in the lesioned PTBI brains.** We have tried several different operators for brain cutting, using both Parrafin embedded, and cryostat-sectioned tissue…we purchased a new cryostat, from non-DOD funds. We seem to have adequate quality, with the cutting work of Mr Julio Diaz, and have employed an experienced Cuban volunteer, trained in histology in Cuba, to help with histology.

A description of work to be performed during the next reporting period.

See table 1. We will complete the cell counts for aim 1. and aim 4 , as limited by time on the confocal and stereology microscopes in the MP.we have hired extra people, see above, to achieve this.
We will finish the last 4 Cartesian diver Microrespirometer animals, during the next quarterly period.
Weare currently performing the aim 2 studies, using Fluid Percussion injury, and will be finished in 2 weeks.
We have a student to do the remaining spleen lung and Liver histology, during the next quarter also, for aim 3.

Administrative Comments (Optional) - Description of proposed site visits and participation in technical meetings, journal manuscripts in preparation, coordination with other organizations conducting related work, etc.

We have presented several abstracts, from these studies for the National Neurotrauma meeting, in Phoenix, AZ in July 2012. –See appendix. We have also submitted 4 abstracts from this work, for the Military Health System Research Symposium MHRS/ATACCC meeting in Ft Lauderdale, in August, 2012…. see appendix. —but only 2 were accepted.we declined to attend the ATACCC meeting, since the attendance costs, ~$700 per person, are too high for us to cover off this award.

Reportable Outcomes

Four abstracts for National Neurotrauma Society, 2012, two abstracts for Military Health System Research Symposium (MHSRS) formerly known as: ATACCC, were possible solely due to work funded by this grant. Four University of Miami graduates could be employed by this grant for the FY2012. Two of those have since moved on to pursue higher education. The other two continue to receive employment via the

grant. In addition this year a University of Miami Medical Student from the class of 2013 has taken a year off to participate in the research supported by this grant, it will earn him a Medical Degree with Research Distinction, further the funding has helped two University of Miami undergrads and a Miami Dade County Public School (MDCPS) high school student obtain Honors distinction in Neuroscience and International Baccalaureate respectively. This grant has allowed establishment of the novel PBBI model at the Miami Project to Cure Paralysis.

Conclusion

All 3 were similar to vehicle, at high doses for this PBBI model. Surprisingly, no differences were significant from vehicle treated injured animals, with this severe PBBI model. The differences between PFCs on PPBI-induced neurodegeneration (based on flurojade B counts) in whole brain have not been significant (Fig 1-2). However further analysis of data to outline the therapeutic zone and effect of PFCs within this zone will be crucial for drawing any conclusions. Glucose use, after PBBI, note that glucose use is reduced about 10 fold (Fig 12-13), by the PBBI injury (vehicle) and this appears to be ameliorated significantly, by Oxycyte (~3 fold improvement) data for the other 2 PFC's is awaited. With respect to the effect of 2 PFC's on VO_2 (brain tissue oxygen consumption) after PBBI we cannot yet report apparent robust improvement as seen in other TBI models previously, but these studies represent only 4 animals per group (Fig 14). The studies with two doses of PFC compared to one dose suggest that 2 doses of Oxycyte confer no more benefit than a single dose, in this closed TBI –Hypoxia paradigm (Fig 4-6). We will await the result of the studies with the other two PFC's. The presence of PFCs Oxycyte and Perftec reduces the number to cells with loss of membrane integrity following in vitro stretch injury from 43 to 31.5 and 28.7 percent respectively (Fig 15-16). The TEG data has major implications. If validated, in rats (ongoing) and TBI injured human blood, it suggests Perftec should not be used for TBI, based on the current data (Fig 8-11).

Respectfully Submitted,
R Bullock
10/01/2012.

Appendices

Abstracts for the National Neurotrauma society meeting., July 2012.

1. PENETRATING BALLISTIC-LIKE BRAIN INJURY INDUCES HYPERFIBRINOLYTIC COAGULOPATHY: A STUDY OF THROMBOELASTOGRAPHY IN RAT MODEL
Julio C. Simon[1], Clayton Jackson[1], Ross Bullock[1], Shoji Yokobori[1], Shyam Gajavelli[1] C.J.Bidot[1], C. Bidot Jr[2], Markus Spurlock[1], Lai Yee Leung[3], Frank C. Tortella[3]

2. EFFECT OF PERFLUOROCARBON OXYGEN TRANSPORTERS, ON COAGULATION: IMPLICATIONS FOR TBI
CJ.Bidot; C. Bidot Jr.; S. Gajavelli; M. Johansen; S. Yokobori; W. Jy; YS Ahn and R. Bullock

3. PERFLUOROCARBON EMULSION IMPROVES CEREBRAL TISSUE OXYGENATION AFTER PENETRATING BALLISTIC-LIKE BRAIN INJURY IN RAT

Clayton Jackson[1], Ross Bullock[1], Shoji Yokobori[1], Shyam Gajavelli[1], Christine Bomberger[1], Michelle Zeiden[1] Lai Yee Leung[2], Frank C. Tortella[2]

4. PENETRATING BALLISTIC-LIKE BRAIN INJURY INDUCES HYPERFIBRINOLYTIC COAGULOPATHY, IN THE RAT MODEL: A STUDY OF THROMBOELASTOGRAPHY.

Julio Simon, Clayton Jackson, Ross Bullock, Shoji Yokobori, Shyam Gajavelli, C Bidot, C Bidot Jr, Markus Spurlock, Lai –Yee Yeung, Frank Tortella.